REPORT

of

COMMITTEE ON THESIS

THE undersigned, acting as a committee of the Graduate School, have read the accompanying thesis submitted by Felix Schneiderhan for the degree of Master of Science. They approve it as a thesis meeting the requirements of the Graduate School of the University of Minnesota, and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science.

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A HISTOLOGICAL INVESTIGATION OF THE INFECTION PHENOMENA OF LOOSE SMUTS OF WHEAT AND BARLEY

A Thesis submitted to the graduate faculty of the University of Minnesota by Felix J. Schneiderhan in partial fulfillment of the requirements for the Degree Master of Science.

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INTRODUCTION

The loose smuts of cereals have for a long time occupied the attention of pathologists and agronomists. While the biology and remedial measures of the covered smuts have been cleared up through the work of Prevost[23], Kühn[20], and Brefeld[6], the biology of the loose smuts and their treatments have been developed in very recent times through the researches of Brefeld[6], Herzberg[12], Jensen[15], Appel and Riehm[21], in Europe and Kellerman and Swingle[19] in this country.

The loose smuts of barley and wheat have sometimes been considered diseases of minor importance, but the losses sustained do not justify this view. According to the report of Germany's Chamber of Agriculture[2] 1905, an epidemic of both smuts caused a loss of 50% in the province of Hannover, while in 1906 another serious epidemic occurred in other provinces of Prussia and in general throughout Germany. Reports of losses of from 5 to 10% have been common in Wisconsin and Minnesota during recent years[9].

The disease is spread through the seed and cannot be detected until the plant heads out. This fact, together with the common practice of importation and distribution of new varieties has made loose smuts almost coextensive with cereal culture. Even though infection in some localities may be very slight, the aggregate loss is usually great. The difficulty of treatment, moreover, discourages the use of preventive measures.

HISTORICAL

Life History and Description.

Before 1888, the cereal smut fungi were considered as a single species known both as Ustilago carbo (B.C.) Tul. and Ustilago segetum (Bull.) Ditt. In 1888, Brefeld[5] observed thatspores of a barley smut sent to him from Japan produced no conidia on germination. Those which he had previously collected in Germany, on the other hand, had produced conidia. He also found smuts of wheat and barley in Germany, which did not produce conidia and named them U. hordei Brefeld, but did not then distinguish between them. In the same year, Jensen[14] concluded from inoculation experiments, that the loose smuts of barley, wheat and oats, were varieties of the same species. His conclusions, however, were not at that time justified. He based the separation on the fact that he had obtained a larger percentage of infection when he dusted wheat kernels with wheat smut spores than when he

had dusted them with barley smut spores or with oat smut spores.

Kellerman and Swingle[19], 1890, on the basis of spore germination studies, divided U. carbo into U. tritici and U. nuda which do not produce conidia, and U. avenae and U. hordei which normally produce conidia. The classification of Kellerman and Swingle[19] is generally accepted today. A more detailed history and a complete synonomy of the loose smuts of wheat and barley is given by Stakman[24].

Maddox[22], of Launceston, Tasmania, produced the first evidence of floral infection of Loose Smuts of wheat and barley in 1895. Hori[13], in Japan, 1907, again demonstrated floral infection. Brefeld[6] in 1903, Hecke[11] in 1905, Wakagawa[13] in 1907, Broili[7] and Lang[20] in 1910 gave conclusive evidence of floral infection. Hecke[11] and Lang[21] gave the histological details of embryo infection in barley.

A complete description of the smutted heads

and characteristics of the spores of both loose smut fungi are given by Kellerman and Swingle[19] and by Stakman[24]. The latter publication also discusses the germination of spores in various media and gives an accountof the vitality of the spores. Herzberg[12] described the differences in the colonies of U. nuda and U. tritici on carrot agar.

Treatments.

Jensen[14] in 1888 concluded that the spores of the loose smuts of wheat and barley rested over on the surface of the seeds or within the hulls of barley, and devised his first hot water treatment, which consisted of treating seed for 2 to 3 minutes in water at 52° C. There was no apparent reduction of smut. After a long series of experiments carried on from 1889 to 1895 Jensen[16] declared, that the best hot water treatment for loose smuts, was to soak the grain for 4 hours in cold water and after allowing the

soaked grain to stand for 6 hours longer in wet sacks, to treat for 5 minutes in water at 52½° C.

For the treatment of loose smut of barley, Kellerman and Swingle[18] in 1889, advised soaking for 4 hours in cold water and, after allowing the grain to stand in wet sacks for 4 hours longer, to dip in water at 52 to 53° C for 2 minutes. Freeman and Johnson[9] used the Jensen hot water treatment with good results. They recommend the following treatment:-

Barley--Soak 5 hours then dip in water at 52°C for 15 min.
Wheat " " " " " " " " 54° " 10 "

According to Appel and Riehm[3], many other attempts have been made to devise control methods for the loose smuts of wheat and barley. Among these are, destroying loose smut heads in the field, selection of large kernels, selection of resistant varieties, the Dauerbad treatment which is a soaking of the grain for 8 to 10 hours in water at 40° C, and the hot air treat-

ment. None of these with the possible exception of the last two has proven of practical value.

The chemical treatments for loose smuts present the latest possibilities. Johnson[17], working on treatments for Helminthosporium gramineum (Rabh.) with grain that was infected with loose smut of barley, showed that soaking in a formaldehyde solution, 1 to 320, for 2 hours, reduced the smut to a mere trace, while check plots showed from 8 to 10% of smut. Appel and Riehm[3] in 1911 showed that copper sulphate was ineffective because of its lack of penetration. They carried on experiments with .1% and .2% solutions of mercury bichloride and found, that while infection was not perceptibly reduced by the use of the .1% solution, the .2% solution applied for 2 hours caused an elimination of the smut. The viability of the seed was greatly reduced by these treatments.

Physiology of Spore Germination.

Appel and Riehm[2] have made an exhaustive study

of the thermal relations of U. nuda and U. tritici. The time required for germination in relation to temperature was determined as follows:-

	9-11°C	14-15° C	22-23° C	27-28°C	29-30C
U. nuda	24-48 hrs.	12-24 hrs.	8-12 hrs.	4-5hrs.	4-6hrs.
U.tritici	21-48 "	6-8 "	2-4 "	4-5 "	6 "

Furthermore, they found that the minimum temperature at which spore germination occurred was 6 to 10° C., the optimum temperature was 26 to 29°C., while the maximum temperature was from 33 to 34° C. After 12hours exposure at 36° C., the spores of both species still germinated. At 42° C. the spores of U. nuda were killed after an exposure of 2 hours, while U. tritici spores still germinated after 4 hours at this temperature. The mycelium from pure culture will withstand an exposure to water at 36° C. for 12 hours and only be slightly retarded in growth. The mycelium was killed after an exposure in water at 48° C for 5 minutes.

STATEMENT OF PROBLEM

If the chemical treatments as reported by Appel and Riehm[3] and Johnson[17] are consistently effective, the hyphae of the loose smuts must lie rather superficially in the seed, or a 2 hour soaking in the chemical solutions would not reach them. This investigation was undertaken to determine, if possible, the exact histological relationship of the hyphae to the seed structures, in order to correlate the facts obtained with the chemical seed treatments.

It is obvious that hyphae in the endosperm of a grain, could be destroyed only by such severe treatment, that the embryo itself would probably be killed. The application of chemicals presents the possibilities of an ideal treatment, but these are limited by the location of the hyphae. A more detailed knowledge of the location of the hyphae and the mode of infection

are therefore of great importance in the further development of the methods of treatment, especially in the light of the recent results of Appel and Riehm[8], and of Johnson[17].

METHODS

The first inoculations were made on Turkey Red wheat grown in the Minnesota Cereal Disease Nursery. Later in the season the work was limited to Minn. No. 105 barley and Minn. No. 169 wheat. The spore material was gathered chiefly from wheat and barley varieties at the Minnesota Experiment Station. Only such heads in which the membrane had already been ruptured were used, in order to duplicate as nearly as possible, the actual condition of the spores in normal field infection.

The heads of plants to be inoculated were classified according to the maturity of the flower. Five classes were made as follows:

Stage A_g^I	Stamens still green, head usually one half to one third still within the boot, stigmas straight, lying between the stamens.*
Stage A_y	Stamens yellow and frequently extruded. Anthers dehiscing, stigma slightly spreading or straight, head usually one third within the boot.
Stage B	Head usually exserted four inches or more. Glumes generally open, ovary swollen from above, irregularly hemispherical. Stigmas plumose and retrorsed. Anthers still dehiscing.
Stage C	Glumes closed, stigma collapsed, ovary about one third mature size, slightly elongated below.

*See plate page 41

Stage C-D — Fruit about one half to one third mature size. "Milk stage".

Stage D — Fruit almost full grown but green and soft. "Soft dough stage."

The same technique used in hybridizing work, was employed in the preparation of the heads for inoculation. The upper and lower spikelets were removed, together with the middle floret of the remaining spikelets in wheat, and the middle row of spikelets in barley.

Three methods of application of spores were used:-

(a) The dry spore method.

(b) Spore suspension method for individual flowers.

(c) Spore suspension method for general inoculation.

In the dry spore method, only those spores were used that would easily shake out on gently tapping

the smutted head. The spores were placed in an insect powder blower, the metal tip of which had been flattened so as to form a very small opening. With this thin edge, the glumes were easily opened, where upon one puff from the blower generally sufficed to darken the stigma and ovary with spores. Besides the blower method, a camel's hair brush and a forceps were used to apply the dry spores. This method was also very effective, but slower, with greater chances of injury to the flower. For the dry spore method, it was found that stages B to C were most easily inoculated because the glumes were often partly open.

In the spore suspension method, enough spores to give a dark color to water, were mixed and applied by means of a fine pointed glass pipette. This method spread the spores not only on the stigmatic area but also on the base of the ovary.

The third method consisted simply in drenching the entire head with a spore suspension. The dark-

ening of the glumes, indicated that they were filled with the suspension.

After inoculation, the heads were covered either with paraffin bags ordinarily used in hybridizing work, tissue paper, or by test tubes which were plugged with cotton after the heads were inserted.[I.]

Every inoculated head was gathered in four sections. The periods of gathering were approximately as follows:

No.I. Twenty-four hours after inoculation.

No.II. Forty-eight " " "

No.III. Seventy-two " " "

No.IV. When seeds had become mature.

Collections I. to III. inclusive, were killed and then preserved in alcohol. Collection IV. was kept in dry seed form.

When the inoculated heads were covered with test tubes, the question arose whether or not the abnormal

I. See Plate page. 40

condition would influence the rate of development of the growing embryo. Heads so subjected were exposed to a saturated atmosphere because the transpired water from the heads soon collected in drops on the inner surface of the tube. Ten heads of Minnesota No. 105 barley and ten heads of Minnesota No. 169 wheat were used. They were not inoculated with smut. Most of the ovaries in each head were in the A stage with the stamens still green. The wheat heads were perhaps slightly more nearly uniform in age than those of barley and were about one half exserted from the boot.

The heads from A to D were not covered with tubes, those from E to J were covered with tubes which were to be removed at intervals as shown in the table. A daily observation of the 10 plants was made at 2 P.M. Since there is always some difference in the degree of maturity of the top, middle and bottom of individual heads, these regions were considered separately and are designated in the tables as T.(top)

M.(middle), B.(bottom). The heads were covered at the first date given in the table. The time of removal of tubes is marked by an X drawn through the rectangle.

Table I

Comparative Maturity Test of Minn. No 105 Barley

17

	July 9	10	11	12	13	14	15	16	17	18	19	20	21	22
A	A_B	A_Y	A_Y-B		B-C	T. B-C M. C-D B B-C	T B-C M D B B-C	B-C B B-C	B-C D C-D	D D C-D	D D C-D	D D C-D	D D D	—
B	A_B	A_Y	A_Y	No Records Taken	B-C	T. B-CD M. C-D B B-CD	T. B-CD M. C-D B. B-CD	D D C-D	D D CD	D D D	— — —	— — —	— — —	—
C	A_B	A_B-A_Y	A_Y		B-C+	T. C-CD M. C-D B C-CD	T. C-CD M. C-D B. C-CD	C-D D C-D	CD-D D CD	D D D	— — —	— — —	— — —	—
D	A_B	A_B-A_Y	A_Y		B-C+	T. C-CD M. C-D B. C-CD	T. C-CD M. C-D B. C-CD	C-D D C-D	C-D D CD-D	D D D	— — —	— — —	— — —	—
E	A_B	A_Y	A_Y		A_Y-B	T B M. B-CD B B	T. B M B-D B B-CD	B B-D B-D	C B-CD B-CD	C C-D CD-D	C C-D D	D C-D D	D D D	—
F	A_B	A_B-A_Y	B		B	T B M B-CD B B	T B M. B-CD B. B	B C-D B	B D B	D D C-D	D D C-D	D D C-D	D D D	—
G	A_B	A_Y	A_Y-B		A_Y B	T B M B-C B B	T. B M. B-CD B. B	B B-CD B	B B-CD B	B B-CD B	B-C B-CD B	B-C BC BC	C-D C-D BC	D D CD
H	A_B	A_B-A_Y	A_Y		A_Y B	B	T. B M. B B. B	B B-C B	B B-C B	B B-C B-C	B B-C B-C	BC BC BC	C-D C-D C-D	CD D D
I	A_B	A_Y	A_Y		A_Y-B	A_Y B	T. B M. B B. B	B B-C B	B-C B-C B	B-C B-C B	B-C B-C B	B-C B-C B	C-D D C-D	D D D
J	A_B	A_Y	A_Y		A_Y-B	A_Y B	T. B M. B B. B	B B B	B B B	B B B	B B B	C-D C-D B-C	C-D C-D BC	D D CD

Table III

Comparative Maturity Test of Minn. No 169 Wheat.

		July 14	15	16	17	18	19	20	21	22	23	24	25	26	27
A	T	A_g	A_g	A_y	A_y	B	B-C	B-C		C-D	C-D	C-D	C-D	D	—
	M.	A_g	A_g	A_y-B	A_y-B	B	B-C	C		D	D	D	D	D	—
	B	A_g	A_g	A_g	A_y	A_y	B-BC	B-C		C-D	C-D	C-D	D	D	—
B	T	A_g	A_y	A_y	A_y-B	B	B-C	C		C	C-D	D	—	—	—
	M	A_g	A_y	A_y-B	B	B	C	C		C-D	D	D	—	—	—
	B	A_g	A_g	A_g-A_y	A_y	A_y	B-C	C		C	C-D	D	—	—	—
C	T	A_g	A_g	A_y	A_y	A_y-B	B-C	B-C		C	C-D	C-D	C-D	D	—
	M	A_g	A_g	A_y	A_y-B	A_y-B	B-C	B-C		C	C-D	C-D	D	D	—
	B	A_g	A_g	A_g-A_y	A_y	B	B-C	B-C		B-C	B-C D	C-D	C-D	D	—
D	T	A_g	A_y	A_y	A_y	B	B-C	B-C	To Key	B-C	B-C	D	—	—	—
	M	A_g	A_y	A_y	A_y-B	B	C	C		C	D	D	—	—	—
	B	A_g	A_g	A_g-A_y	A_y-B	B	B-C	B-C		B-C	C-D	D	—	—	—
E	T	A_g	A_y	A_y	A_y-B	B	B-C	B-C	1	C	C-D	C-D	D	—	—
	M	A_g	A_y	A_y-B	A_y-B	B	B	B-C		C-D	D	D	D	—	—
	B	A_g	A_g	A_g-A_y	A_y	A_y	B-BC	B-C		C-D	D	D	D	—	—
F	T	A_g	A_g	A_y	A_y-B	B	B	B		B-C	C-D	C-D	C-D	C-D	D
	M	A_g	A_g	A_y-B	A_y-B	B	B	C		C-D	D	D	D	D	D
	B	A_g	A_g	A_g-A_y	A_y	A_y	A_y-B	B-C		C-D	D	D	D	D	D
G	T	A_g	A_y	A_y	A_y-B	B	B	B	Record is	C	C	C-D	C-D	C-D	D
	M	A_g	A_y	A_y-B	A_y-B	B	B	B		B-C	C-D	C-D	D	D	D
	B	A_g	A_g	A_g-A_y	A_y	A_y	A_y-B	B-C		C-D	C-D	C-D	C-D	C-D	D
H	T	A_g	A_y	A_y	A_y-B	B	B	B-C		C-D	D	—	—	—	—
	M	A_g	A_y	A_y-B	A_y-B	B	B-C	B-C		D	D	—	—	—	—
	B	A_g	A_g	A_g	A_y	A_y-B	B	B-C		C-D	D	—	—	—	—
I	T	A_g	A_y	A_y-B	A_y-B	A_y-B	B-C	B-C	No	B-C	C	C-D	D	—	—
	M	A_g	A_y	A_y-B	B-C	B	B-C	C		D	D	D	D	—	—
	B	A_g	A_g	A_y	A_y-B	A_y-B	B-C	B-C		B-C	C-D	D	D	—	—
J	T	A_g	A_y	A_y	A_y	A_y	B-C	B-C		B-C	C-D	C-D	D	—	—
	M	A_g	A_y	A_y-B	A_y-B	A_y B	B-C	B-C		D	D	D	D	—	—
	B	A_g	A_g	A_g-A_y	A_y	A_y	B	B-C		B-C	D	D	D	—	—

In both wheat and barley the general effect of the tubes is to delay the development. This was especially noticeable in the barley. There is a decided difference in the time of ripening of the top, middle and bottom of the heads. This offers a longer period for infection.

Exact conclusions in regard to the comparative results are not safe, in view of the small number of heads used and the variation in individual heads. The general effect of delay in the covered heads in reaching D stage was however quite noticeable.

The tables show also, that the average length of time to develop all of the young and immature ovaries in an uncovered head in the A_g stage, to the D stage, under the weather conditions of the experiment, was about 11 days for wheat and 10 days for

barley. The individual variation in the time of maturing together with the earliness and lateness of the different varieties, extends the period of susceptibility to loose smuts of these cereals.

Fixing and Staining.

Three fixing fluids were used, Flemming's medium solution, Juel's Solution and Carnoy's Fluid. Most of the material was fixed for 24 hours in Flemming's solution, after which it was washed for 10 hours and then run up to 70% alcohol. When Juel's Solution was used, the material was fixed for 12 hours, after which it was washed at one hour intervals in 50% alcohol and then run up to 70% alcohol. In Carnoy's Fluid the material was fixed for 3 hours after which it was washed three times at one hour intervals in 95% alcohol and then run back to 70% alcohol. All of the material was preserved in 70% alcohol until used.

Three stains were used, Triple Stain, Gram's Stain and the Dilute Safranin Stain, which consists of

staining 6 seconds in iron alum, 6 seconds in haematoxylin and 7 minutes in dilute safranin. Of these, Gram's Stain proved to be the best for histological purposes, the Dilute Safranin Stain was better for the detection of cytological details, although the discovery of hyphae with the latter stain could only be made with the high power of the microscope. The best results were obtained with the above stains when the material was fixed in Flemming's solution. It was also observed that the age and kind of material had a direct influence on the staining results.

Pure Cultures.

For pure culture work, beerwort, carrot, beef, cellulose and synthetic agars were used. Of these, the beerwort and carrot agars were by far the best. The cultural characters corresponded with those given by Hersberg[12] and Brefeld[6], the differences between the two fungi being very clear. An attempt to produce spores was made by transferring cultures to beef agar

and cellulose agar, but without success. Both developed very slowly on these media. The colonies developed to a diameter of from 5 to 10 m.m. after 15 days. In all the cultural work, U. nuda grew more rapidly than U. tritici.

Sectioning.

The preserved wheat material, (Collections I. to III. inclusive) hardened, probably on account of the action of the alcohol on the wheat glutins and could be cut only with difficulty. The barley material on the other hand could be easily sectioned.

Hecke[11], Brefeld[5], Maddox[22] and Freeman and Johnson[7] found that the period of maximum glume opening, at which time the feathery stigmas are extruded, was the optimum for floral infection. In the present investigation, most of the inoculations were made at this stage . On examining sections it was observed that the spores germinated more vigorously on the stigmas than on the surface of the ovary. Pollen

grains were frequently infected by the germ tubes. On account of this fact it seems reasonable to suppose that the method of entrance of the fungus may possibly be the same as that of the pollen tubes.

Penetration of the ovary wall is probably more difficult than that of the stigmatic hairs and infection would probably not be as likely to occur in this region. Furthermore, the greater chance of lodging, the presence of nutrient sugar solution and the presence of more moisture on the stigmas, furnish more favorable conditions for infection.

The mature seeds of the No. IV. collection were sterilized with mercury bichloride (1 to 1000) and placed on sterile blotters in a germinator. Some seeds were allowed to germinate for 24, some for 48 and some for 72 hours. After several attempts it was concluded that embryos from seeds which had been in the germinator for 48 hours, were most suitable for sectioning. The embryos were detached with sterile scalpels and tweezers, killed in Flemming's medium killing fluid,

cut 10 microns thick and stained with Gram's Stain or with the Dilute Safranin Stain.

On sectioning the embryo of a germinating wheat kernel (IV.Collection) that had not yet broken out of the seed wall, (44 hours old) the infection of the embryo was observed, the entire embryo being apparently infected. The infection hyphae appear as short, irregular, unbranched and much knotted strands in the cells of the scutellum. As a rule, the hyphae are unbranched except when they follow the vascular bundles. The greatest length of a single hypha observed was 15 to 16 microns. The hyphae are apparently both inter and intra cellular. In the very young stages, the hyphae are apparently multinucleate with very few septations. There is no close uniformity in the size of the hyphae, which seems to depend upon their age and the tissue in which they grow. The average diameter is about 4 microns, they range from 3 to 9 microns. Those immediately beneath the absorptive cells of the scutellum

are larger in diameter than those farther in the embryo. The diameter of the hyphae found in the growing point is usually from 3 to 4 microns.

All of the slides examined show that the hyphae develop best in the upper part of the scutellum. Whether this is due to the close proximity to the seat of diastatic action in the absorptive cells or to the main vascular bundle in this region, was not determined.

The formation of clusters of hyphae two and three cells beneath the absorptive layer was characteristic. In these clusters, the hyphae appear to be twisted and knotted about one another, in contrast to the more regular and thread-like development in the growing point and its base. Hecke[11] observed these clustered hyphae and designated them as "Mycelmeester". He observed that the hyphae only rarely penetrated the intercellular spaces of the absorptive cells of the scutellum. The writer has observed this phenomenon very frequently.

Hyphae were also found in the extreme end of

the enveloping leaf sheaths. In one case a hypha was found in an apical cell of the leaf sheath. Furthermore, long and well developed hyphae were found in the root tissue. Both of the above observations show that the hyphae are not necessarily localized in the stem growing point and the region between it and the scutellum as previously reported by others.[11,20] Mycelium was frequently found in the vascular bundle in the upper part of the scutellum.

Hyphae were also found in the attached seed coats. These hyphae are identical in appearance with those previously observed, the only difference being that the hyphae found in the seed coats did not stain as deeply as those inside of the embryo. The aleurone layer appears to limit the hyphal development in the seed coats. Hyphae were found along this layer and to a distance of from 3 to 4 cells outside of it. In a few cases the hyphae were seen entering the embryo through the more or less disintegrated aleurone layer near the embryo. The hyphae were also found in the layer outside of thenucellus and in the nucellar layer itself. No hyphae were found in the endosperm tissue of any of the seeds.

Methods of Embryo Infection.

The fact that hyphae have been found in the young growing point does not necessarily prove that infection of the embryo had already taken place previous to germination. There are three possibilities of infection, viz:- during intraseminal development of the

embryo, during extraseminal development i.e. after germination of the seed, or during both of these periods. If infection occurs during extraseminal development it is possible that the hyphae rest over in the seed coats. The exact method of embryo infection is important in the correlation of seed treatments. If the hyphae overwinter in the seed coats and are localized in this region until spring, then a much more severe chemical treatment would be necessary than that used for such smuts as Tilletia foetens, but it would not need to be as severe as it would if the hyphae were in the embryo.

The penetration of the hyphae through the embryo end of the aleurone layer as described above is suggestive of the original method of entrance.

The discovery of hyphal threads in the seed coats outside of the aleurone layer obviously suggests the possibility that loose smuts of wheat and barley overwinter partly in the seed coats. Furthermore, if infection of the embryo occurs before the period

of grain maturity, it might take place from the seed coats outside of the aleurone or probably from the nucellus which contains more nutrient material than the other layers. The phenomenon in this latter case would be very similar to th at described by Freeman[11] in his work on the intraseminal infection of Lolium temulentum .

The tissue of the scutellum contains far more hyphae than does the stem growing point. The peculiar formation of hyphal clumps may possibly be a resting adaptation of the fungus. No such phenomenon has been observed in the growing point. Abundant mycelium in the scutellum, which, at the time of seed ripening is the major portion of the young plant, leads to the conclusion that infection takes place at the time of intraseminal development, and the hyphae rest over not only in the seed coats but also in the scutellum of the embryo. The fact that as early as 44 hours after germination an embryo, even before it has broken out of the seed coats contains hyphae in both

scutellum and growing point, is good evidence that infection of the embryo has taken place before the beginning of germination. Initial infection of the embryo probably occurs through the absorptive cells of the scutellum as indicated by the greater number of hyphae in its upper part.

FACTORS INFLUENCING PRIMARY INFECTION.

After inoculation, as described above on pages 12 and 13, approximately 85% of the heads were covered with test tubes because it was believed that a fairly saturated atmosphere such as that inside of the test tubes would be conducive to spore germination and subsequent infection. Furthermore, the cotton plug would eliminate all outside contamination. From the results obtained both in infection and in the number of seeds that set, it is evident that certain factors existed that were not favorable, since a lower percentage of infection was generally obtained than reported by previous investigators. An attempt was subsequently made to determine the principal

factors influencing primary infection.

Phototropism

It was thought that the light shining through the test tubes might have some influence on the direction of germ tube growth if their response was positively phototropic. To determine if possible the response of germ tubes, hanging drop cultures of modified Cohn's Solution were made in Van Tieghem cells . These cells were kept at room temperature under a box with a narrow slit cut through one side at the height of the drop. Counts were made after two days to determine the percentage of germ tubes growing toward and away from the light. The following table shows the direction of germ tube growth in relation to light.

TABLE SHOWING THE DIRECTION OF GERM TUBE GROWTH IN RELATION TO LIGHT.

		Positive	Negative
U. Nuda	Cell I	99	101
	" II	100	99
	" III	80	60
	" IV	29	25
	" V	47	58
	Total	355	343
U.tritici	Cell I	97	83
	" II	94	96
	" III	104	96
	" IV	99	81
	" V	92	91
	Total	486	447

The above table shows that phototropism, at least in nutrient solution, plays little part in the direction of the growth of germ tubes. In many cases the germ tubes branched in such a manner that one branch grew toward the light while another branch grew in the opposite direction.

Hydrotopism

The air inside of the test tubes was probably fairly saturated because the transpired water could

not readily escape. If the germ tubes were positively hydrotropic they would probably grow away from the surface of the ovary. To determine the nature of germ tube response to a fairly saturated atmosphere, carrot agar was poured into 5 petri dishes and allowed to stand until fairly dry. A small circular piece of agar was then removed from the middle of the plate, forming a shallow cup which was nearly filled with sterile distilled water. A streak culture was made about one-fourth of an inch from the edge of this cup. The colonies near the water developed better than those farther from it, and in general, the growth seemed to be toward the water.

Another experiment was carried on in test tubes, the bottoms of which were stuffed with cotton and saturated with water. About half-way up, an agar colony was placed on the clean wall of the tube and allowed to grow. The tube was slanted at an angle of about 15°. The mycelium grew toward the water. Colonies of both smuts growing on carrot agar in a fairly saturated atmosphere developed aerial

mycelium. It appears therefore, that the germ tubes of the loose smut fungi are positively hydrotropic and this may have had some influence in the direction of their growth in the test tubes.

Thermal Relations

According to Herzberg[12] the maximum temperature for spore germination is 33 to 34° C. while the optimum is 25 to 29° C. During some of the days on which inoculations were made, the temperature was 32 to 36° C. in the shade. This high temperature outside, together with a saturated atmosphere and a dead air space on the inside of the tube, possibly caused a temperature sufficient to kill a large number of the spores. The following experiment was carried on to determine the difference in temperature on the inside and on the outside of the test tubes. The plants were grown in the green house and were used at the B and C stages. (See page 11)
A thermometer was inserted into the tubes which covered the heads. Two such tubes, A and B, were used. Besides

this, the temperature in a tube without a plant was taken, in order to determine the effect of the saturated atmosphere. The following table shows the results.

TABLE SHOWING TEMPERATURE RELATIONS
INSIDE AND OUTSIDE OF TEST TUBES .

A- B Test tubes containing heads of grain.

C " " " no head " "

D Check temperature in the greenhouse at same level as A, B, and C.

Varying Temperatures in One Day

A

4/22/15	A	B	C	D
9 A.M.	20°C.	20°C.	20°C.	20°C.
10 " "	24.5	25	23	22
11 " "	27.5	28	25	22
12 P.M.	27.5	28.5	25	22
1 " "	32	32.5	29	27

A

4/22/15	A	B	C	D
2 P.M.	36	36	34	32
3 " "	37	37	35	32.5

Table Showing Temperatures on Various Days

B

4/22/15	A	B	C	D
	36°C.	36°C.	34°C.	32°C.
4/23/15	32	32	28	25
5/10/15	42	37	36	33
5/12/15	38	42	37.5	35

From the above tables it can be concluded that the temperature inside of the tubes is on an average several degrees higher than the outside air temperature. The saturated atmosphere causes a difference of 2.9° C. as is shown in the difference between A,B and C. The exact relation at higher temperatures has not yet been determined because the temperature up to date has not been high

enough. Such temperature relations as are recorded above, undoubtedly had a deleterious effect on spore germination and account for the low percentage of infection obtained.

Period of Incubation

In the sections of II collection (48 hours after inoculation) it was apparent that a much larger percentage of the spores had germinated than in the I collection (24 hours after inoculation). It seems therefore, that a two-day period of incubation is more advisable than a one-day period under field inoculation conditions. This is an important consideration in the technique of inoculation.

LOCAL INFECTION

From experiments carried on in connection with this work, no evidence was obtained that local infection could take place either on the very young embryo plant or on leaves that have emerged 3 or 4 inches above ground. Twenty-five leaves of wheat and barley were inoculated with pure culture material of U. tritici and U. nuda respectively, and

placed under a belljar. At the end of 5 days the leaves were examined and the mycelium was found growing along the surface, but infection had not taken place.

Embryos that had been growing 44 hours were detached from the seeds and the scutella were covered with loose smut spores taken directly from smutted heads. These embryos were placed on the surface of sterilized starch paste (2 parts wheat starch to 5 parts of water) in petri dishes, with the scutella on the medium. Both barley and wheat embryos grew vigorously on this medium. After 4 days the embryos were examined. The spores germinated but caused no infection. The results cannot however, be considered conclusive since contamination by imperfect fungi may have interfered with the development of the smut. The same technique was used in inoculating embryos of wheat and barley with pure culture material of their respective loose smuts. Mycelium developed on the surface of the scutellum but no infection resulted.

CONCLUSIONS

I. Histological proof of initial floral infection has not been demonstrated in this investigation. There is evidence to show that the stigmatic surface presents more favorable conditions for infection than the ovary wall.

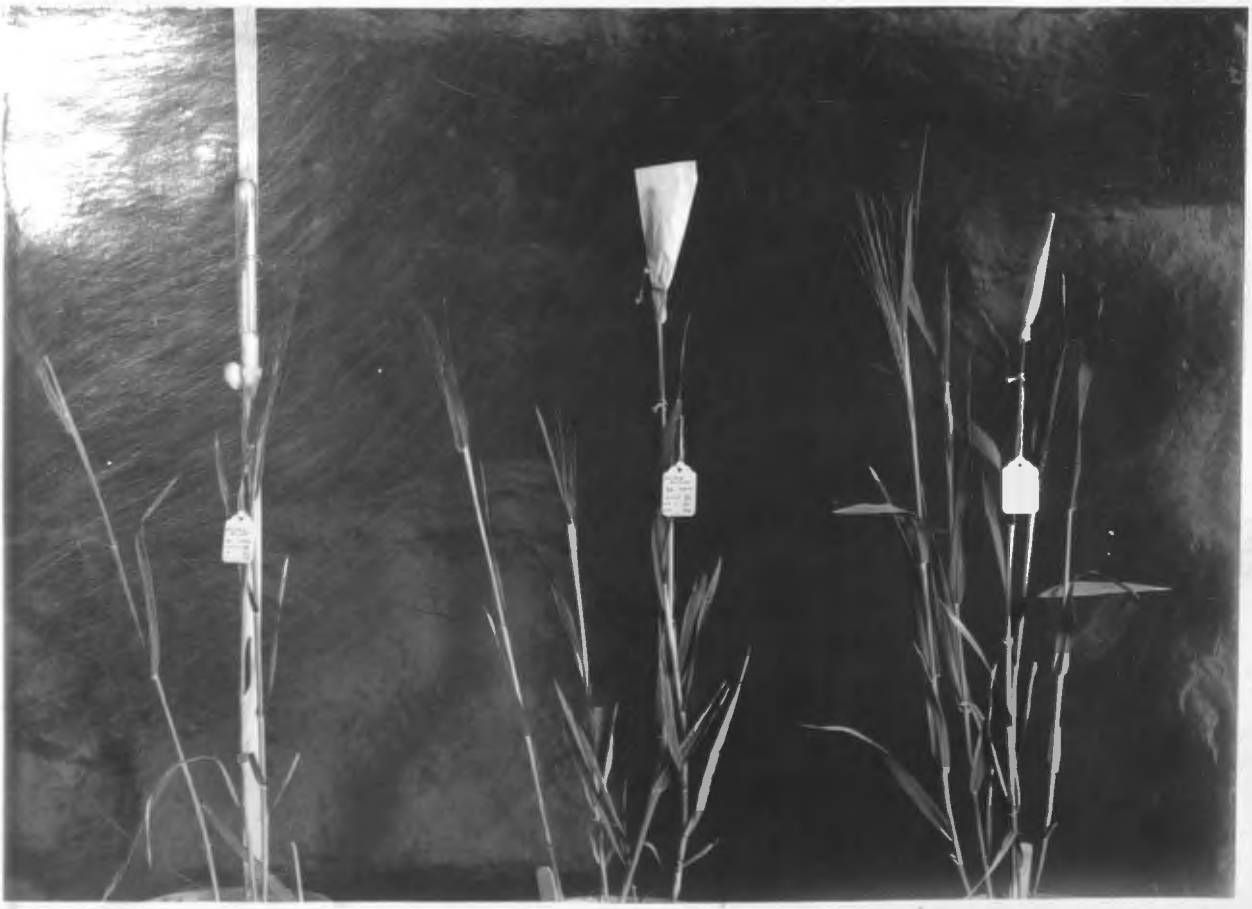
II. Hyphae of the loose smuts of wheat and barley have been found in the seed coats, but not in the endosperm.

III. The histological details of embryo infection were observed. They were essentially as Hecke has described them. In addition, however, the loose smut hyphae were found not only in the scutellum and stem growing point, but also in the apical region of the leaf sheaths and in root tissue.

IV. Infection of the embryo probably normally takes place before the seed is mature.

V. Local inoculation on both leaves and young embryos did not result in infection.

Plate I



A B C

Methods of Covering Inoculated Heads

A --- Test Tube Method

B --- Paraffin Bag

C --- Tissue Paper

Stage Aβ

Stage Aγ

Stage B

Stage C

Stage C-D

Stage D

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